VARIABLE INTER-ANNUAL RELATIONSHIPS BETWEEN T-CELL MEDIATED IMMUNITY AND INDIVIDUAL TRAITS IN WHITE STORKS

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Summary.—*Variable inter-annual relationships between T-cell mediated immunity and individual traits in White Storks.*
Aims: The PHA skin-testing technique has become a widespread way of studying the ecological and evolutionary implications of T-cell mediated immunity (CMI) in birds. Several breeding and individual traits have been found to be related to CMI. However, it is not known how consistent these relationships are over the years. A four year study was conducted on the CMI response of White Stork *Ciconia ciconia* nestlings to evaluate this point.
Location: Dehesa de Abajo, Puebla del Río, Sevilla, España.
Methods: T-cell mediated immunity was measured by the PHA skin-testing technique in 1999, 2001, 2002, and 2003, as well as seven parental or individual variables for each nestling: laying date, clutch size, brood size, sex, age, brood hierarchy, and body condition.
Results: The relationship between CMI and individual traits over the years depended on the studied variable. Some of them, when significantly related, showed a consistent relationship (*e.g.* body condition), while others did not have any relationship (*e.g.* age) or even contrary trends (laying date) depending on the year.
Conclusions: These results highlight the need of including the temporal scale into ecological immunology for a better understanding of the relationships between CMI and individual traits.
Key words: *Ciconia ciconia*, immune system, inter-year variability, individual traits, nestlings, PHA-test.

Resumen.—*Variaciones interanuales en las relaciones entre la respuesta inmune celular y otros caracteres individuales en la Cigüeña Blanca.*
Objetivos: La técnica de la Fithohemaglutinina (PHA) se ha convertido en un método común en el estudio de las implicaciones ecológicas y evolutivas del sistema inmune celular (CMI) en las aves, y se han identificado varias características reproductivas y de los individuos relacionadas con el CMI. Sin embargo, no sabemos cómo de consistentes son estas relaciones encontradas con el CMI en una escala temporal. Este punto fue evaluado estudiando el CMI de los pollos de Cigüeña Blanca *Ciconia ciconia* durante cuatro años.
Localidad: Dehesa de Abajo, Puebla del Río, Sevilla, España.
Métodos: Se midió el CMI de los pollos con la técnica de la PHA en 1999, 2001, 2002 y 2003, así como siete variables de cada individuo: fecha de puesta, tamaño de puesta, tamaño de pollada, sexo, edad, jerarquía dentro de la pollada, y condición física.
Resultados: La relación entre el CMI y los caracteres individuales a través de los años dependió de la variable estudiada. Algunas variables, cuando estuvieron relacionadas significativamente con el CMI, mostraron relaciones consistentes (*e.g.* condición física), mientras que otras no mostraron ninguna relación (*e.g.* edad) o incluso correlaciones opuestas según los años (fecha de puesta).
Conclusiones: Estos resultados ponen de manifiesto la necesidad de incluir la escala temporal en aspectos de ecología immunológica para un mejor entendimiento de las relaciones entre el CMI y los caracteres individuales.
Palabras clave: Caracteres individuales, *Ciconia ciconia*, pollos, sistema inmune, test de la PHA, variación interanual.

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INTRODUCTION

The PHA skin-testing test (Goto *et al.,* 1978) was commonly used in poultry science as a method to evaluate, *in vivo*, the T-cell mediated immune response (CMI) of birds, which is one of the main components of the immune system of vertebrates (Roitt *et al.*, 1996). After the pioneering paper of Saino *et al*. (1997), however, this technique has become a common measure of the T-cell-mediated immunity (CMI) of wild birds in many ecological and evolutionary studies. Examples of this are studies on the relationships between CMI and the evolution of avian life-history traits (Martin *et al*., 2001; Tella *et al*., 2002), breeding time (Sorci *et al*., 1997; Moreno *et al*., 1998), clutch size (Tella *et al*., 2000a), brood size (Sorci *et al*., 1997; Ilmonen *et al*., 2003), brood hierarchy (Roulin *et al*., 2003), nestling growth (Soler *et al*., 2002), breeding sociality (Møller *et al*., 2001; Tella *et al*., 2001), arrival date (Møller *et al*., 2004), dispersal (Møller *et al*., 2004), sex (Fargallo *et al*., 2002), senescence (Lozano & Lank, 2003), secondary sexual traits (González *et al*., 1999), sexual displays (Soler *et al*., 1999), or mating decisions (Johnsen *et al*., 2000).

Most recent studies conducted upon CMI have been undertaken during a single year, and those studies conducted during more than one year have not focused on the variability of the relationship between CMI and other individual traits (*e.g*. Saino *et al*., 1999; Møller, 2002). Thus, it is not known how these potential associations between CMI and individual traits behave along an ecological time scale. Through a four-year study on White Stork *Ciconia ciconia* nestlings, CMI and several variables previously related to CMI in birds were measured (*i.e*. laying date, clutch size, brood size, brood hierarchy, sex, age, and body condition). The aim here was not to study why differences among years could arise, but rather to assess whether relevant relationships hold across years.

MATERIAL AND METHODS

CMI of nestlings was studied in a colony of White Storks («Dehesa de Abajo», Puebla del Río, Sevilla, Spain) during the breeding seasons of 1999, 2001, 2002, and 2003. All the nests studied are sited in *ca*. 4 m-high wild oli-

ve trees, allowing the assessment of clutch size from the ground with the help of a mirror added to the end of a pole. Two or three visits to each nest were undertaken during the laying and incubation period for recording complete clutches. Nestlings were ringed, and aged according to the formula provided by Negro *et al*. (2000): age = 5.068 + (0.117 * wingchord length). Laying date was calculated for each nest as the day of hatching of the oldest chick minus 33 days (corresponding to the length of the incubation period). Nestlings were sampled for CMI between 37 and 67 days of age (median 53 days), that is, in the last part of their nestling period. This is the period when CMI is commonly measured in the majority of avian studies (see compilation in Tella *et al*., 2002), when the nestling immune system is assumed to be most developed. Moreover, most of nestling mortality in the studied colony is concentrated in ages under 20 days, and particularly under 10 days (Jovani & Tella, 2004). In this way, sampling nestlings above 37 days of age, one could be confident that brood size at PHA-test reflects the number of chicks fledged from each nest.

T-cell mediated immune response was assessed by subcutaneously injecting 0.1 mL of 2 mg/mL PHA-P (Sigma) in phosphate buffered saline (PBS), in a marked site of the interdigital membrane of the left foot. The thickness of the marked site was measured three times with a digital micrometer (to the nearest 0.001 mm) before injection and 24 hours after injection. Since measurement repeatability was high for a random sample of 100 nestlings both on their first (intraclass correlation coefficient, r = 0.99, $F_{99,200}$ = 1417.6, $P < 0.001$), and second measure (r = 0.99, $F_{99,200}$ = 1135.3, $P < 0.001$), CMI was calculated as the difference between the mean of the second minus the mean of the first measure (Smits *et al*., 1999). The sex of each nestling was determinated by molecular procedures (Ellegren 1996) using DNA extracted from the cellular fraction of a few drops of blood.

Brood hierarchy was scored in three categories: oldest, medium, and youngest nestlings. Single nestlings were classified as the oldest ones because brood reduction in White Storks usually affects the youngest nestlings (Sasvári *et al*., 1999; *pers. obs*.). Body mass of nestlings was measured (to the nearest 25 g) at the time

of PHA injection and once again 24 h later, coinciding with the measure of the skin swelling. For each chick, a body condition index was calculated as the residuals from a Model II regression between the $\log_{10}$ of body mass and the $\log_{10}$ of the wingchord length (Green, 2001). The minimum, but not the mean, body mass of the two measures was used to reduce the confounding effects of undigested meals on the body mass of nestlings (Tella *et al.*, 2001).

CMI of nestlings is potentially influenced by both common parents and rearing conditions (Brinkhof *et al.*, 1999; Christe *et al.*, 2000; Soler *et al.*, 2003; Tella *et al.*, 2000b). The *r* intraclass correlation coefficient was used to estimate the fraction of total variance on CMI attributable to sharing the same nest. This coefficient was calculated as the division between the variance of CMI among siblings, and the total variance of CMI in the population using the «variance components» tool in SPSS 11.0. Significance of *r* coefficients was calculated with a one-way ANOVA -see Lessells & Boag (1987) for further details-. Within years, the nest of rearing had a statistical effect on the CMI of nestlings (see results), thus siblings were considered as non-independent analysis units. However, among years, nestlings born in the same nests were considered as independent analysis units since they were not similar for means ($r = 0.000$, ANOVA: $F_{47,62} = 0.872$, $P = 0.685$) or for the standardized mean of CMI for each nest and year (intraclass correlation coefficient $r = 0.000$, ANOVA: $F_{47,62} = 0.878$, $P = 0.677$). Therefore, for all the analyses, the family (group of nestlings born on the same nest and year) was used as a random factor in generalized linear mixed models (GLMMs) with normal distribution and identity link function, using GLIMMIX macro of SAS for avoiding pseudoreplication (see Tella *et al.*, 2001).

First, the relationship of each measured variable on CMI was tested through univariable tests for each year and for each variable separately. Bonferroni correction was not applied because the aim was to show to what extent single year studies conducted on a reduced number of individual traits could produce significant univariable relationships. In a second analysis, multivariable models were produced where the year and its interactions with the other variables were fitted to the model to asses the consistency of the relationship between CMI and individual variables across years, while controlling for the expected covariance among the explanatory variables. Non-significant effects ($P > 0.05$) were removed in a backward procedure starting with a saturated model with all the variables fitted to CMI data. Because CMI could show circadian rhythms (Navarro *et al.*, 2003), the discrete variable «time» was also fitted into the model to control for the moment of the day when CMI was measured (morning/afternoon). A small sample of nestlings with external deformities in legs and bills were excluded from the analyses to avoid masking the results with the inclusion of potentially sick individuals. All tests were two tailed.

RESULTS

CMI was measured from 121, 126, 155, and 44 nestlings, from 54, 54, 76, and 24 nests in 1999, 2001, 2002, and 2003, respectively. Univariable analyses gave a puzzling picture of relationships between CMI and each individual trait (Fig. 1). Relationships of CMI with laying date became significant in two years, with opposite trends. A lower CMI was found for females in one year and nearly significantly in another one. The same happened for the positive correlation of CMI with body condition. Significant relationships were found for clutch size in one year. Moreover, nearly significant statistical effects were found for brood size (two years) and brood hierarchy (two years).

In the multivariable analysis, however, only a positive correlation of body condition ($F_{1,235} = 8.61$, $P = 0.0037$), and the interaction between laying date and the year of study (Laying date: $F_{1,235} = 9.82$, $P = 0.0019$; Year: $F_{3,235} = 11.45$, $P < 0.001$; Laying date * Year: $F_{3,235} = 10.88$, $P < 0.001$) were retained in the final model (Table 1). The rest of the variables (clutch size, brood size, sex, age, brood hierarchy, and time), and their interactions with year did not reach significance (all P-values > 0.24) to be retained into the model. Note that these discrepancies between the univariable and the multivariable analyses are expected in studies such as ours, because of the covariance among individual variables tested. For instance, univariable relationships between body condition and
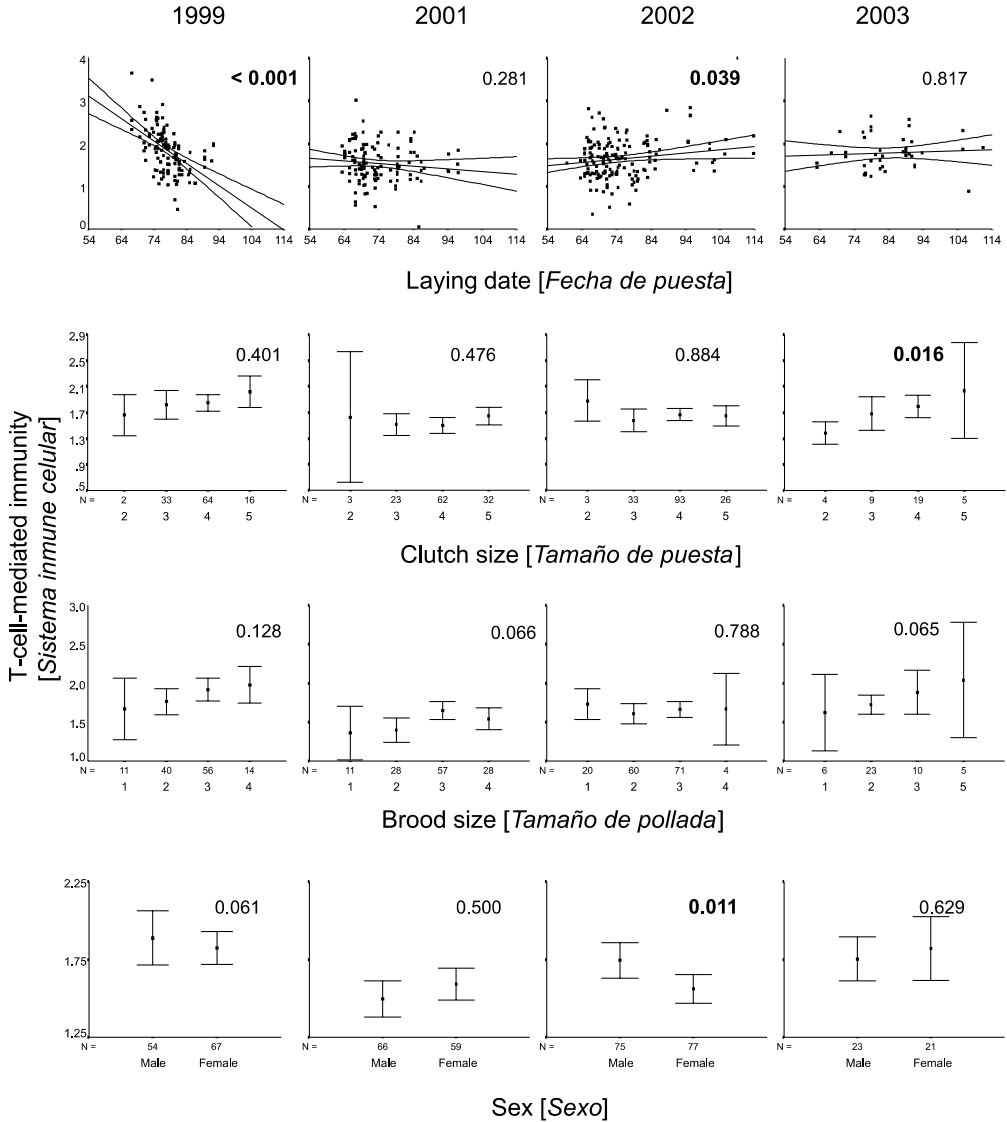
FIG. 1.—Univariable relationships between individual traits and T-cell-mediated immune response of White Stork nestlings. Note that error bars depict 95% confidence interval of means. *P*-values of univariable GLMMs with family as a random factor are indicated, with significant results in bold. See text for statistical significance of these relationships in multivariable analysis.
[Relaciones univariables entre las características individuales y la respuesta inmune celular de los pollos de Cigüeña Blanca. Las barras de error muestran el intervalo de confianza al 95% para las medias. Se indica el valor de la P de los GLMMs univariables utilizando la familia como factor aleatorio, con los resultados significativos en negrita. Ver texto para la significancia de estos resultados en análisis multivariable.]
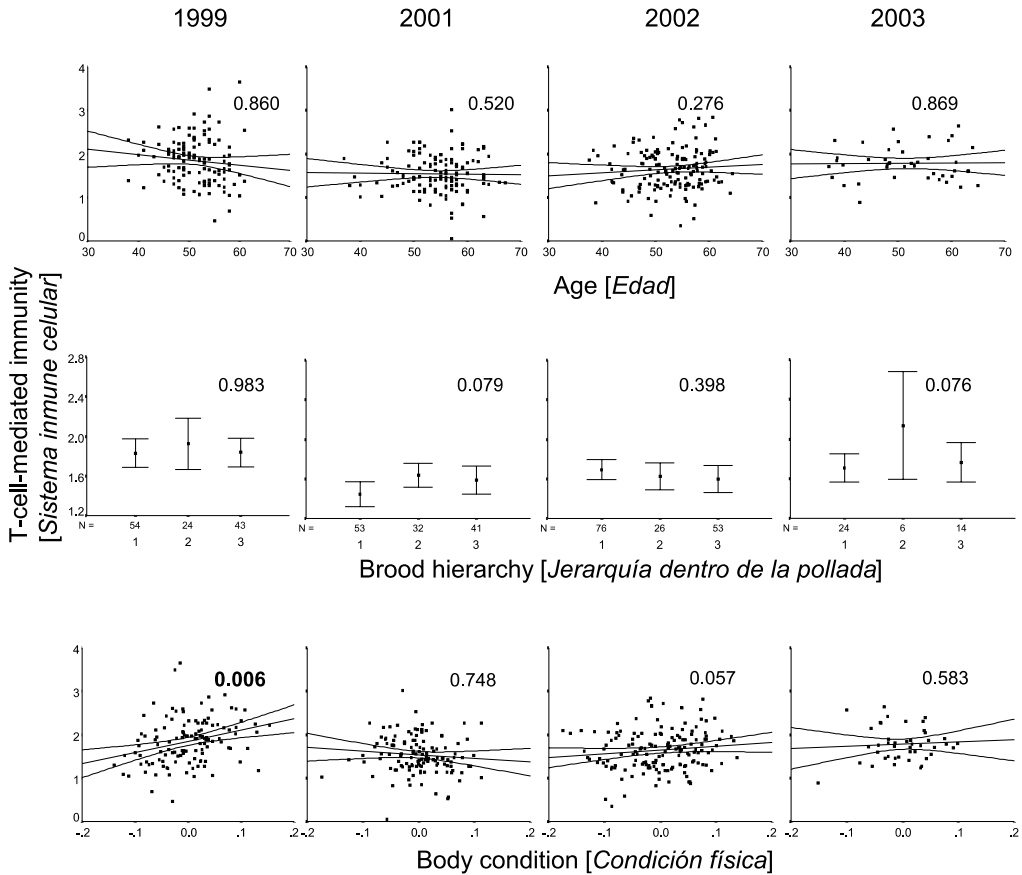
CMI was significant in one year and nearly significant in another, but only body condition - but not its interaction with the year of study - entered in the final multivariable model. In fact, family had a significant statistical effect on CMI both in the multivariable analysis ($Z = 0.093$, $P < 0.001$), and for the three years with big enough sample size to be tested separately (*i.e.* 1999, 2001, 2002; all P-values < 0.02). Moreover, repeatability analyses showed the same result (1999: $r = 0.528$, ANOVA: $F_{53,67} = 3.503$, $P < 0.001$; 2001: $r = 0.354$, $F_{53,72} = 2.201$, $P = 0.0010$; 2002: $r = 0.268$, $F_{75,79} = 1.717$, $P = 0.0092$).

DISCUSSION

The consistency of the relationships between CMI and individual traits through years was variable. Some variables did not show any relationship with CMI (*e.g.* age), while others had a significant correlation in some years but not in others (*e.g.* clutch size, sex and body condition), or only marginal statistical effects in some years but not others (*e.g.* brood size and brood hierarchy). Despite the high variability of these associations, there were some relationships that were more consistent over time than others. Body condition showed a more consistent positive correlation with CMI, and family appeared as a relevant factor. These two variables have been shown to be relevant on CMI in previous observational (Tella *et al*., 2001) and experimental (Alonso-Alvarez & Tella, 2001; Brinkhof *et al*., 1999; Soler *et al*., 2003; Tella *et al*., 2000b) single-year studies, and here we confirm that they may constitute a relatively consistent pattern over years. Moreover, females had similar or lower CMI than males. This

TABLE 1

Results of the multivariable mixed model for the relationships between parental and individual traits of White stork nestlings and their T-cell-mediated immune response. *SE* = Standard Error. df = Degrees of Freedom. See text for further details.
[Resultados del modelo multivariable mixto del efecto estadístico que relaciona las variables parentales e individuales de los pollos de Cigüeña blanca sobre su sistema inmune celular. ES = Error Estándar. df = Grados de Libertad. Ver texto para más detalles]

Effect [Efecto]	Year [Año]	Estimate ± SE [Coeficiente ± ES]	df [gl]	t	P
Intercept [Intercepto]		1.592 ± 0.590	201	2.70	0.0076
Body condition		1.101 ± o.375	236	2.93	0.0037
[Condición física]					
Year [Año]	1999	4.037 ± 0.950	236	4.25	< 0.001
Year	2001	0.393 ± 0.738	236	0.53	0.60
Year	2002	–0.629 ± 0.660	236	0.95	0.34
Year	2003	0	236		
Laying date [Fecha de puesta]		0.002 ± 0.007	236	0.30	0.77
Laying date*Year					
[Fecha de puesta*año]	1999	–0.050 ± 0.012	236	4.29	< 0.001
Laying date*Year	2001	–0.008 ± 0.009	236	0.89	0.37
Laying date*Year	2002	0.007 ± 0.008	236	0.89	0.38
Laying date*Year	2003	0	236		

result is contrary to previous studies with CMI in adult birds (Moreno *et al*., 2001) and nestlings (Fargallo *et al*., 2002; Tschirren *et al*., 2003) and with the overall lower immune response of domesticated male animals (Grossman, 1985).

Especially relevant was the shift of the relationship between laying date and CMI through years, being negative (also found by Sorci *et al*., 1997 in Magpies *Pica pica*), but also neutral or even slightly positive depending on the year of study. Although still controversial (Norris & Evans, 2000), CMI seems to be constrained by short-term fluctuations in energy demanding functions (Soler *et al*., 2002) and the availability of resources (Lifjeld *et al*., 2002). This implies that different annual environmental conditions affecting CMI (Møller, 2002), or even subtle differences on the timing of «bad condition» episodes within breeding seasons (Christe *et al*., 2001), could produce the variable relationships found here across years between phenology and CMI or other fitness-related traits previously reported (*e.g*. survival Monrós *et al*., 2002). In this sense, nestling white storks have been shown to be sensitive to inter-year variability of weather conditions (Jovani & Tella, 2004), and thus this kind of envi-ronmental variability could affect both the growth and development of the immune system of nestlings, varying in its relationships with parental and individuals' traits among years. Additional research effort is needed for testing this possibility.

These results alert of how important the temporal variability on the relationships between CMI and individual traits could be even on a short time-scale. Therefore, if the results on White Storks are the rule for other bird species, they suggest that previous findings would be seen as identifiers of potential relevant links between CMI and the evolution of certain individual traits, but this does not mean that they must be constant factors operating on the evolution of these traits. In the same way, these results also indicate that failing to find a correlation between CMI and an individual trait in one year does not exclude the possibility that this relationship could be relevant in other years.

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